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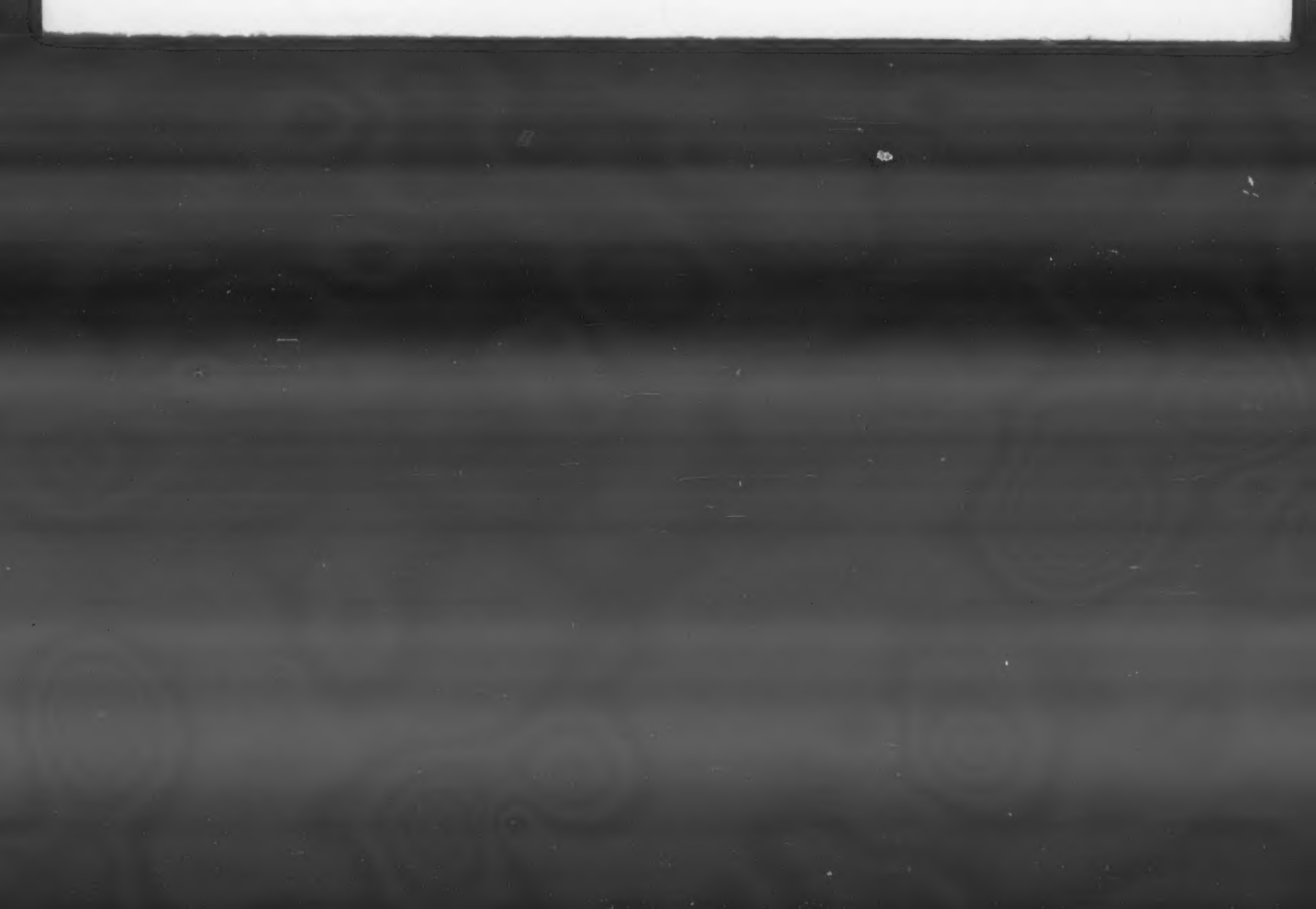

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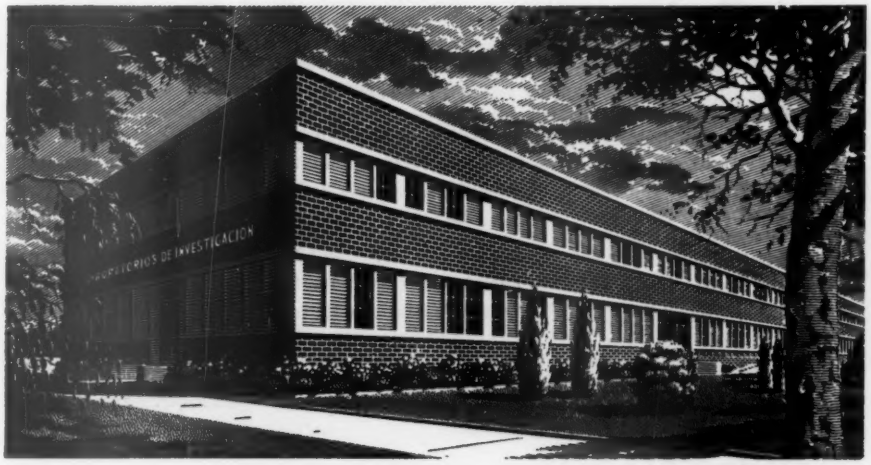
extiende sus actividades industriales al campo de la investigación científica



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1. Knight, V.: New York State J. Med. 50:2173 (Sept. 15) 1950. 2. Herrell, W. E., Heilman, F. R., y Wellman, W. E.: Ann. New York Acad. Sc. 53:448 (Sept. 15) 1950. 3. Blake, F. G., Friou, G. J. y Wagner, R. R.: Yale J. Biol. and Med. 22:495 (Julio) 1950. 4. Perdrup, A.: Ugeskrift for Laeger 112:1089 (Agosto 3) 1950. 5. Pulaski, E. J.: Ann. New York Acad. Sc. 53:347 (Sept. 15) 1950. 6. Bickel, G., y Plattner, H.: Schweiz. med. Wchnschr. 81:1 (Enero 6) 1951.

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*) *Beams, A. J. y Endicot, E. T. Cambios histológicos en hígados de pacientes con cirrosis, tratados con Metionina. Gastroenterology 9:718-735 (Dec.) 1947*

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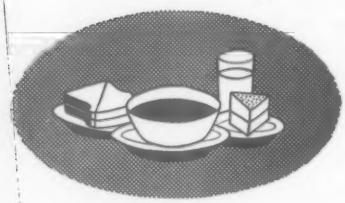

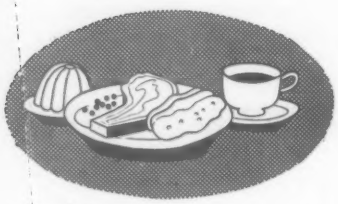

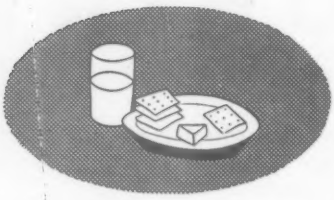

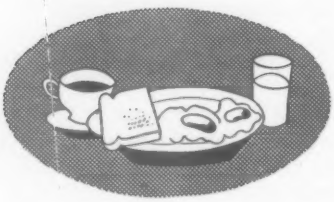

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Vol. III Nº 1

1953

ASOCIACION CIENCIA E INVESTIGACION

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ANGIODIACTIN: A NEW VASODILATOR SUBSTANCE OF THE TISSUES

JUAN CARLOS FASCIOLO

DURING the investigations on the renin content of kidneys it was found that renal extracts contained a substance, which greatly diminished the vasoconstrictor action of hypertensin in a toad preparation. Further investigation led to the identification of a vasodilator substance, whose pharmacological and chemical characteristics differ from those already known. The new substance has been called angiodiactin.

METHODS

Toad vascular preparation: The identification and estimation of angiodiactin was carried out with the Läwen-Trendelenburg preparation using the toad *Bufo arenarum* Hensel. The technic has been already described (1) (fig. 1). The preparation was kept in a state of mild vasoconstriction by epinephrine. 30-60 minutes after the perfusion was started, 40 γ of epinephrine per liter of perfusing fluid were added, a quantity which usually reduces to a half the drop output. After a few minutes the preparation stabilizes and it is ready for use. The sample to be tested is injected in the preparation as shown in fig. 1, vasodilatation being recognized by an increased flow as measured by the drop output. An angiodiactin unit has been defined as the amount, which under these conditions, will produce an increase of 10 drops per minute. Preparation 211* was standardized (contains 2.000 Units per ml) and used for comparing the activities of unknown samples. To compensate for changes in sensitivity of the vascular preparation, the sample to be titrated is injected between 2 standards, as follows: first, the standard, and then, when the effect has

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This work was completed at the Instituto de Fisiologia, Universidad Nacional de Tucumán, Argentina. Present address: Departamento de Fisiologia, Facultad de Ciencias Médicas, Dr. Tomás Perón, Mendoza, Argentina.

* The following angiodiactin preparations are mentioned in this study:
P 211 - 2.000 Units per ml; solid residue 8.4 mg per ml, solvent petroleum ether.
P 191 - 2.000 Units per ml; solid residue 4.9 mg per ml, solvent petroleum ether.
P M3 - 4.000 Units per ml; solid residue 2.7 mg per ml, solvent methyl alcohol.

Received for publication May 14th, 1953.

passed off and the previous flow level or a new plateau has been reached, the unknown sample is tried. Later, another standard dose is injected. Varying the amount of the unknown sample tested, it is possible to obtain a vasodilator action similar to that of the standard. As a rule, vascular preparations show more sensitivity to angiodiactin at the beginning of the experiment, than after two or three hours. Sometimes they become quite unsensitive after this period of time. Seldom they become unsensitive after one or two tests.

Preparation of the sample to be tested: Angiodiactin is kept in solution in acetone, ether, petroleum ether, etc., according to the batch. To test its activity on the toad preparation, the solvent must be evaporated and angiodiactin suspended in the perfusing fluid. A small amount of the solution, usually 0.05 - 0.20 ml, is pipetted in a test tube and evaporated with the aid of a vacuum pump. By heating and shaking the tube, the small remnant is suspended in 10 ml of the perfusing fluid (Hülse solution) *. Most purified samples give transparent or slightly turbid solutions. Solutions are foamy but the purest preparations produce much less foam than the crude ones. To inject in the vascular preparation, 0.05 - 0.20 ml of this solution are pipetted in a small syringe and made up to 1 ml with Hülse solution.

Rat vascular preparation: In order to find out whether angiodiactin has vasodilator action on mammalian vessels, a vascular preparation of rats' hind legs was devised. Rats were killed with a blow on the head. The abdomen was opened and a cannula introduced into the aorta immediately below the origin of the renal arteries. The cannula was connected with a perfusion system similar to the one used in the toad preparation, but Ringer's solution, with or without calcium, was used as the perfusing fluid. The vena cava was dissected and ligated rostrally to the origin of the renal veins. An incision is made now in the vena cava below the ligature, and once the blood and perfusing fluid have drained for a while, an L shaped glass cannula is introduced. The shorter arm is tied in the distal end of the cava, and the larger arm, emerging at the side of the rat, facilitates the counting of the number of drops. With a little practice this preparation can be completed in a few minutes. If desired the preparation can be readily eviscerated.

The perfusion fluid was in every case at room temperature, about 25°C (limits ranged between 22 - 27° C). Perfusion pressure varied from 40 to 60 cm of water, with which drop outputs of about 80 drops per minute were obtained. In most preparations there is some loss of fluid, which, however, does not disturb the determinations. The preparation oedematizes rapidly and after 2 or 3 hours the oedema is quite pronounced. In spite of that the number of drops is not greatly reduced and the preparation shows good sensitivity to vasoconstrictors.

The vasomotor substances are tested in this preparation as already

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* Sodium chloride 6 g
Sodium citrate 5 g
Potassium chloride 0.5 g
Distilled water to 1 liter.

described in the case of the toad preparation. The preparation is usually sensitive to 0.005 - 0.02 γ of epinephrine, and to 0.0005 Units of hyper-

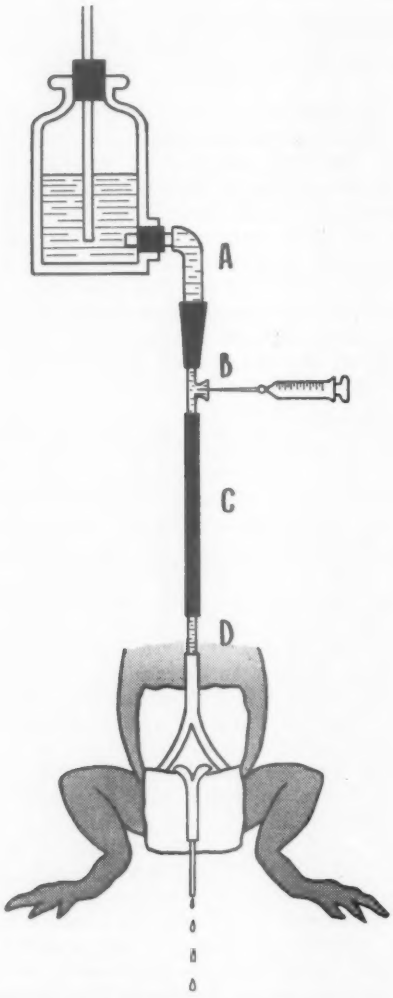

FIG. 1. — *Schematic representation of the device employed for the assay of angiodiactin. Mariotte's flask is filled with the perfusing fluid to which 40 γ of epinephrine per liter were added. Samples were injected (always made out to 1 ml volume) through the T tube with a small syringe. Glass tube A and rubber tube B are wide to avoid obturation of the system by bubbles introduced at the moment of the injection. Tubes C and D are narrow to avoid dilution of the sample.*

tensin *, during the 2 or 3 hours in which it remains in good condition (Table I). To test the vasodilator effect of angiodiactin the preparation was kept in a state of mild vasoconstriction, adding 10 γ of epinephrine per liter to the perfusing fluid. This causes a reduction of flow of about 50 per cent.

Preparation of angiodiactin: After removing the fat, the capsule and the renal pelvis, kidneys are cut in small pieces and ground in a homogenizer.

One volume of 96 per cent alcohol is added, pH brought to 5 - 6, and after extracting for 48 hour at room temperature, the extract is filtered

TABLE I

Sensitivity of a rat vascular preparation to various vasoconstrictor substances

Substance	Doses	Drops per minute			Duration of the effect Minutes
		initial	final	difference	
dl-epinephrine (1)	0.05 γ	102	96	—6	3 (100)
l-epinephrine (2)	0.05 γ	106	82	—24	7 (102)
l-nor-epinephrine (2)	0.05 γ	102	88	—14	5 (102)
Hypertensin	0.0005 Units	88	70	—18	10 (86)

Vascular preparation perfused with Ringer without calcium. Temperature 25°C; perfusing pressure 50 cm of water. Figures in brackets in column 6 indicate the number of drops after the vasoconstrictor effect has passed off.

(1) Epinephrine Rhone Poulenc (racemic).

(2) Laevo epinephrine and nor-epinephrine were kindly furnished by Sterling-Wintrop through the courtesy of Dr. Pi-Suñer.

through a Büchner funnel. The alcoholic filtrate is stored, and the precipitate extracted again with 3 volumes of 96 per cent alcohol, at pH 5 - 6. Temperature is raised to about 70°C for 30 minutes, and after cooling, the solution is filtered through Büchner. The precipitate is discarded and the alcoholic filtrate mixed with the one of the first extraction. Alcohol is distilled out under reduced pressure.

In the watery remnant, on cooling, a milky precipitate appears which contains most of the activity. This precipitate settles down slowly and cannot be easily separated from the rest of the solution. It is extracted twice with equal volumen of petroleum ether**, giving a pale brown solution. In this stage an angiodiactin unit weights about 300 γ.

Petroleum ether is distilled off, and the brown residue extracted under reflux three times with boiling acetone. After cooling a yellow acetonic solution and a voluminous brown precipitate are obtained. The latter is discarded. At this stage the dried acetonic residue has an activity of one unit of angiodiactin per 20 γ of weight.

* A hypertensin unit is the amount which will raise 20 - 30 mm Hg the blood pressure of a 10 kg chloralozed dog.

** Boiling point 40 - 70°C.

Two grams of calcium hydroxide per 10 ml of acetone are added and heated to 45°C while continuously stirring. After cooling to 5°C, the solution is filtered through Büchner. Most of the activity remains in the precipitate. The precipitate is washed out 3 times with cold acetone, which removes some yellow material, and then dissolved with 20 per cent HCl, HCl is added slowly with constant stirring till the solution remains acid. This solution is now extracted three times with a volume of ethyl ether. The ether solution is yellowish and contains approximately 60 per cent of the original activity of the kidney, angiodiactin unit weighing about 4 - 6 γ.

Further purification is achieved by adding to the ether solution a volume of 5 per cent barium hydroxide, which removes much inactive

TABLE II

Effect of boiling on angiodiactin. Toad vascular preparation constricted by epinephrine

	Drops per minute		
	Initial	Final	Difference
2 minutes boiling Acid medium	38	68	+ 30
Original solution	60	88	+ 28
2 minutes boiling Alkaline medium	74	96	+ 22

Two solutions of angiodiactin (P 211) containing 20 Units per ml were boiled, one at pH 2.5 and the other at pH 10. Aliquots of one unit were tested on the vascular preparation.

material, most of the activity remaining in the ether. By evaporating the ether and extracting the residue 3 times with boiling methyl alcohol, most of the activity goes in solution. On cooling to —20°C a white inactive precipitate is formed which is easily removed. Angiodiactin unit weights about 1.5 γ. The purest preparation so far obtained has one unit in 0.7 γ.

CHEMICAL PROPERTIES OF ANGIODIACTIN

Solubility: Angiodiactin is soluble in 95 per cent alcohol, ethyl ether, petroleum ether, acetone and chloroform. Since it can be precipitated from ethyl ether with diluted sodium hydroxide and 5 per cent sodium carbonate, it appears to have an acidic group.

Angiodiactin can also be precipitated from petroleum ether by a 2 per cent sodium hydroxide solution. In the water-ether interfase a milky film is formed which contains almost all the activity. Barium hydroxide also precipitates angiodiactin from petroleum ether solutions.

From acetone solutions most of the activity can be precipitated

with calcium hydroxide. With barium hydroxide only around half of the active substance precipitated. From this precipitate most of the activity is recovered by ethyl ether.

From ethyl ether solutions, barium precipitates much inert material and only a fraction of the active substance.

Thermosensitivity: 2 minutes' boiling at pH 2.5 or pH 10 does not produce loss of activity of angiodiactin solution (Table II).

Biological actions

Vascular action in the toad: Employing the toad vascular preparation, as described under methods, angiodiactin begins to show its vaso-

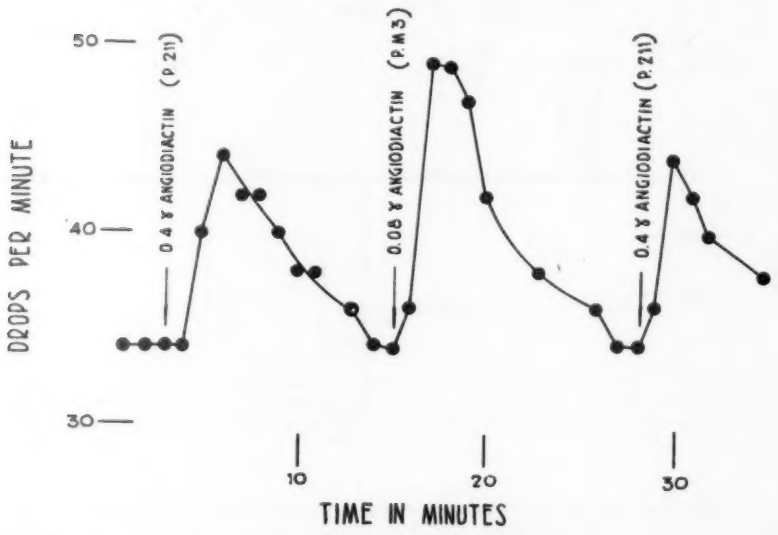

Fig. 2. — *Toad vascular preparation constricted by epinephrine. The action of angiodiactin on the vessels of the toad kept in a state of mild vasoconstriction by epinephrine, is shown by an increase in the number of drops. A rapid vasodilator effect can be seen, followed by a slower return to previous values. Angiodiactin M_3 is far more purified than 211, producing more vasodilator effect with doses 5 times smaller.*

dilator action one minute after the injection, the flow values reaching its maximum in 3 - 5 minutes, to regain the previous level in 15 - 30 minutes (fig. 2). According to the dose employed and the sensitivity of the preparation, these values show a wide range of variation. In some cases it is necessary to wait one hour or more for the vasodilator effect

to disappear. As a rule vascular preparations retain their sensitivity to angiodiactin for hours, and during this period of time the vasodilatation is within limits proportional to dose. However, some vascular preperations loose their sensitivity more rapidly, and in some cases it is not possible to obtain vasodilatation even using larger amounts of angiodiactin (fig. 3).

Less purified angiodiactin preparations have vasoconstrictor effect when tested in the toad, in amounts 4 or 5 times larger than those employed to elicit strong vasodilatation. The vasoconstrictor effect is more prolonged than with epinephrine and rapidly develops tachyphy-

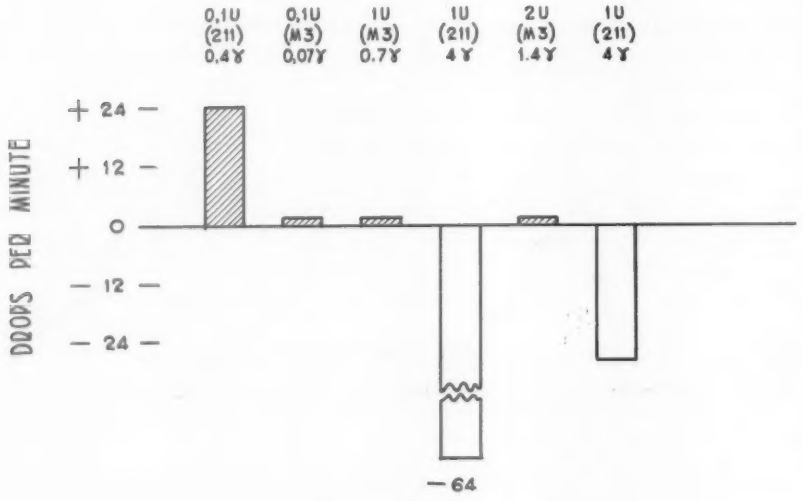

FIG. 3. — *Toad vascular preparation constricted by epinephrine. Figure illustrates a case in which tachyphylaxis to angiodiactin followed a single dose (this seldom occurs). At the beginning the preparation was sensitive to 0.1 Units, but later doses 20 times as large were without effect.*

It can be seen that angiodiactin 211 (1 U.) produced vasoconstriction, but double doses of more purified M_3 were without vasoconstrictive effect.

laxis (fig. 4). More purified preparations have less or no vasoconstrictor effect, as shown in fig. 3.

The action of angiodiactin on the vessels of the toad can be shown in a different way. The toad preparation is perfused with Hülse *without* epinephrine; 0.5 - 1 γ of epinephrine are injected in the preparation and the vasoconstrictor effect measured by the reduction in number of drops per minute. Angiodiactin is now injected in the usual way and 4 mi-

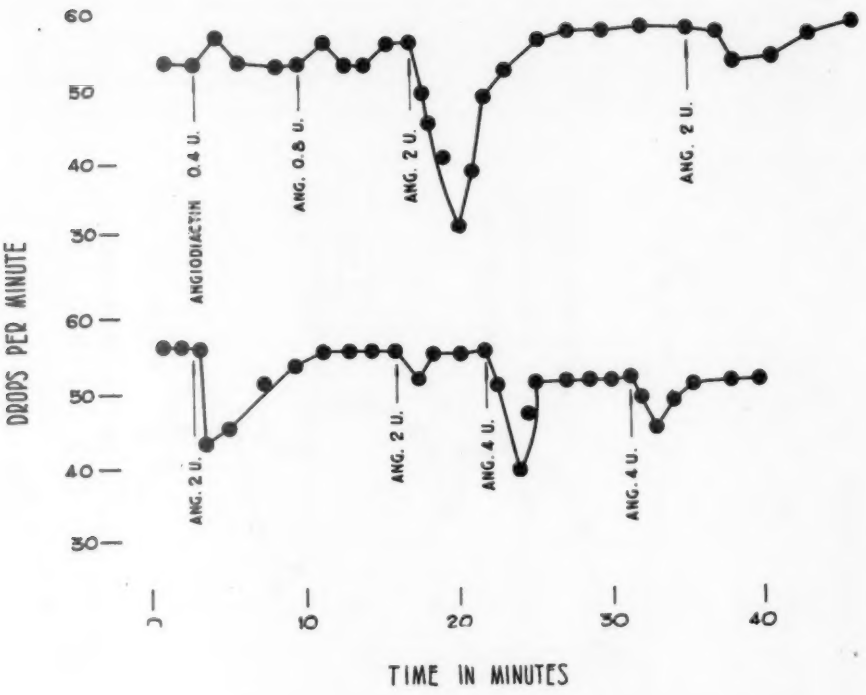

FIG. 4. — *Unconstricted toad vascular preparation. Usually angiodiactin has not effect in the unconstricted vessels as shown in this figure. Larger dosis produce vasoconstriction, but tachyphylaxis develops readily. Preparation 211 was used in this experiment.*

nutes later the vasoconstrictor effect of the same amount of epinephrine is recorded. The reduction in the vasoconstrictor effect of the second dose of epinephrine will measure the activity of angiodiactin. As shown in fig 5, the depressor effect of angiodiactin on the sensitivity of the preparation to epinephrine lasts 20 - 40 minutes.

Angiodiactin can be tested on a toad vascular preparation without adding epinephrine or other vasoconstrictors to the perfusion fluid. In this case the vasodilator action is small or can be absent. Probably this is due to the fact that Läwen-Trendelenburg preparations are in a state of maximal vasodilatation and for that reason are insensitive to vasodilator effects. However it has been possible in some preparations to obtain intense vasodilator effects.

Hypertensin can replace epinephrine to reveal angiodiactin vaso-

dilatation. Hypertensin can be added to the perfusing fluid or tested before and 4 minutes after angiodiactin. It can be seen in figs. 6 and 7 that angiodiactin vasodilatation is more gradual and lasts longer with hypertensin; this keeps pace with the more prolonged effect of hypertensin on toad vessels.

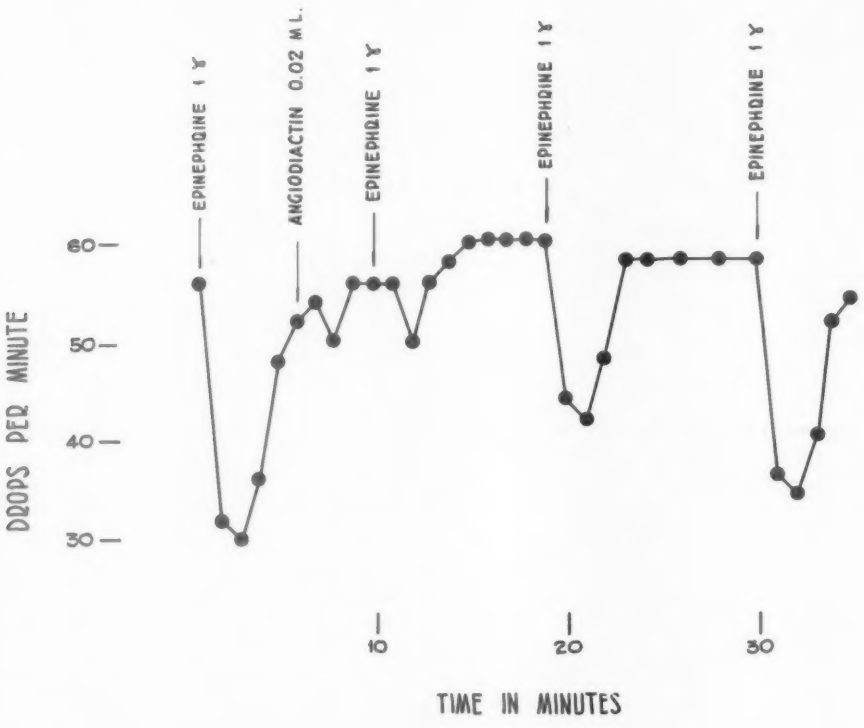

FIG. 5. — *Unconstricted toad vascular preparation. In this experiment the vascular effect of 1 γ of epinephrine was tested before and 4 minutes after the injection of angiodiactin. It can be seen that the vasoconstrictor effect of epinephrine is greatly diminished and even 30 minutes after the injection of angiodiactin the previous sensitivity had not yet been reached.*

Angiodiactin 101 employed in this experiment was not standardized.

Effect of atropine: In order to know whether the action of angiodiactin can be inhibited by atropine, 200 γ of atropine sulfate were injected in a vascular preparation perfused with Hülse fluid plus epinephrine. Atropine caused an increase in the number of drops, which reached the previous level 20 minutes later. At this time 2 Units of

angiodiactin were injected and produced its usual vasodilatation (Table III).

Vascular action in the rat. The vascular action of angiodiactin was tested in the rat employing the technic described under methods. It has been possible to observe vasodilator effects with smaller doses than those used in the toad (0.02 Units, by weight 0.05 γ of P 191) (Table IV).

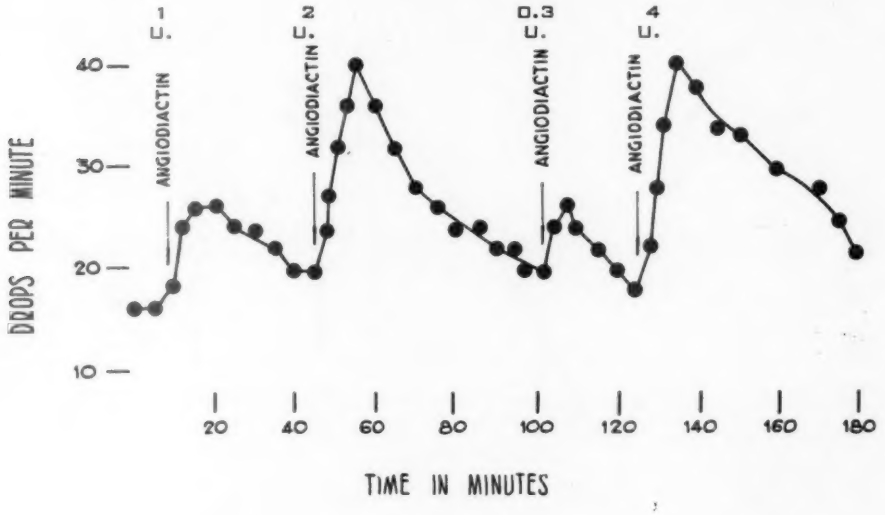

FIG. 6. — *Toad vascular preparation constricted by hypertensin. The vasodilator action of angiodiactin is slower and lasts longer when hypertensin is used instead of epinephrine to contract the vessels (compare the abscissa values with fig. 2). Dilatation obtained with 2 Units, appears to be maximal since 4 Units produced almost the same effect, although of larger duration. Preparation 211 was used in this experiment.*

The rat preparation readily develops tachyphylaxis to angiodiactin. Some preparations were insensitive from the beginning.

Action on arterial pressure. Adult rats anesthetized with nembutal were employed. Arterial pressure was measured in the carotid artery with the technic described by D'Amour and Blood (2) *, heparin being used as an anticoagulant. 200 to 500 Units of angiodiactin were injected intravenously without observing significant changes in arterial pressure.

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* Determinations were kindly done by Dr. Edmundo Albaca.

Similar experiments were conducted in dogs, under nembutal anesthesia, arterial pressure being recorded with a mercury manometer. Intravenous doses of 500 to 5.000 Units suspended in 10 ml saline solution failed to affect significantly blood pressure values.

Action on isolated heart. The hearts of the toad *Bufo Arenarum* Hensel, prepared according to Straub were used. Hearts were sensitive to 0.005 - 0.10 γ of epinephrine. Angiodiactin doses varying from 6 to 10 Units (M_3) were without effect in most preparations.

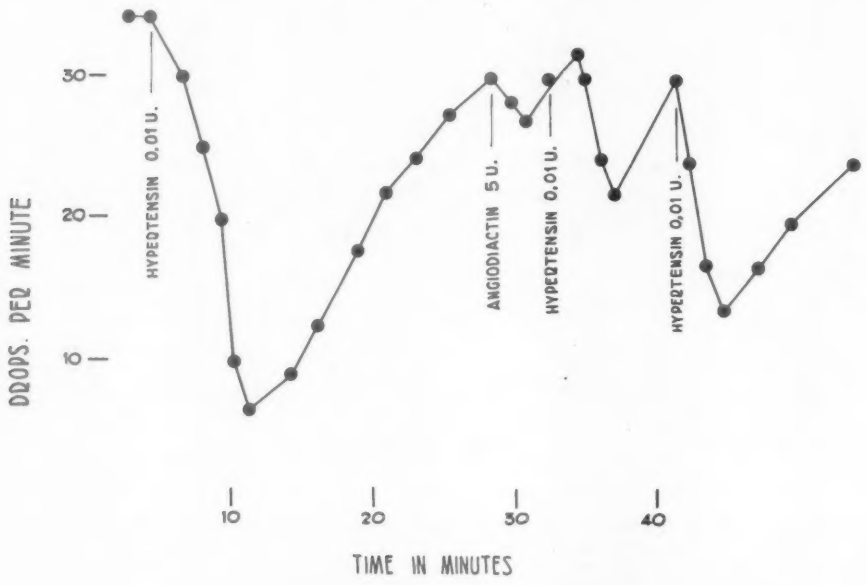

FIG. 7. — *Unconstricted toad vascular preparation. Hypertensin vasoconstriction is greatly diminished after the injection of angiodiactin. Angiodiactin P 191 was used in this experiment.*

Action on isolated heart. The hearts of the toad *Bufo arenarum* rhythmically in oxygenated Ringer solution at 37°C was employed. The preparation displayed inhibition of motility and tone by the addition to the bath of 0.2 γ of epinephrine. Angiodiactin (100 to 400 units, M_3) was without effect.

Toxicity. Two unanesthetized rats were given intravenously (tail vein) 2.000 units of angiodiactin (P 211) suspended in 1 ml of Ringer solution. No immediate effect was observed, nor death or signs of disease in the following days.

Six normal rats were daily injected subcutaneously with 1000 Units

TABLE III

Effect of atropine on angiodiactin vasodilatation. Toad vascular preparation constricted by epinephrine

	Drops per minute		
	Initial	Final	Difference
Angiodiactin 1 U.	66	76	+ 10
200 γ atropine	62	96	+ 34
Angiodiactin 2 Units	74	94	+ 20

Angiodiactin was injected before and after atropine. Between the injection of atropine and the second dose of angiodiactin 24 minutes had elapsed.

TABLE IV

Effect of angiodiactin on the rat vascular preparation, constricted by epinephrine

	Drops per minute			Duration of the effect Minutes
	Initial	Final	Difference	
0.02 U. angiodiactin	74	84	+ 10	5
0.02 U. angiodiactin	74	84	+ 10	8
0.02 U. angiodiactin	78	86	+ 8	6

Vasodilator effect obtained with 3 succesive doses of angiodiactin. 5 γ of epinephrine per liter were added to the Ringer perfusing the rat preparation. 0.02 Units of P 211, weighing 0.05 γ were used.

of angiodiactin * dissolved in 0.2 ml of sunflower oil, during six days. Systolic arterial pressure was measured every day in the tail with the pletysmographic method ([3]). No significant changes in blood pressure, body weight or health were observed. In another series doses were doubled without observable effects.

* P 191 was used. The standardization is only approximate.

Occurrence in the body. As already mentioned, angiodiactin has been prepared using the kidney as the starting material. The yield is of about 150.000 Units per kg.

Using the same method (purification was not carried out to the last steps) it has been possible to extract from the liver a substance with the same vasodilator action of angiodiactin on the toad vascular preparation. Probably both substances are the same, but so far we have not tried all the chemical and biological tests of identification.

Discussion

There are in the tissues several substances described by Feldberg and Schiff (4) under the name of tissue hormones, and by Gaddum (5) as vasodilator substances of the tissues. Some of these substances, such as histamin, acetylcholine and adenosine are well identified chemically, while others such as kallikrein (6), P substance of Gaddum (7), vagotonin (8), angioxil (9), Major and Weber substance (10), prostaglandin (11), vesiglandin (12), and others (13, 14, 15) are not.

Many of these substances were not satisfactorily identified and for that reason it is difficult to do a comparative study of them in relation to angiodiactin. In some cases such as in the so called "Frühgift", the active substance appears to be adenylic acid (16).

More recently Shorr, Zweifach and Furchgott, 1945 (17) have described V.D.M., a vasodilator substance present in liver.

Angiodiactin can be differentiated from other vasodilator substances present in the tissues not only by its solubility characteristics but also by its biological properties.

Differentiation from histamin. Histamin produces a fall in arterial pressure of the anesthetized dog, while angiodiactin, even if used in high dosage, does not show hypotensive effects. Histamin contracts the isolated ileum of rabbit while angiodiactin has no action on it. Furthermore, histamin when tested on the vascular toad preparation constricted by epinephrine, under the conditions described above, does not produce vasodilatation (doses ranging from 0.02 - 2 γ). Higher doses usually cause vasoconstriction.

Differentiation from acetylcholine. Acethylcholine produces a fall in arterial pressure and contracts the isolated rabbit ileum, both actions being absent in the case of angiodiactin. Furthermore, acetylcholine even in high dilutions stops the isolated toad heart while angiodiactin, as already mentioned, has no effect.

Differentiation from adenosine and adenylic acid. Adenosine and adenylic acid * showed vasodilator action on the toad vascular preparation constricted by epinephrine (doses ranging from 1 to 20 γ, were tried), but their action is weaker than angiodiactin. For instance, in one case 10 γ of adenylic acid produced a vasodilatation resulting in a flow increase of 4 drops per minute, while the same amount of angiodiactin

* Kindly furnished by Dr. Luis F. Leloir.

on the same preparation caused an increase of 36 drops per minute. Similar results have been obtained with adenosine.

The effect on arterial pressure also allows to differentiate angiodiactin from adenosine or adenylic acid, the latter two being hypotensors.

Differentiation from other substances. Kallikrein ([6]), Werle's kallidin ([18]), P substance ([7, 19]), Lange and Felix ([20, 21]) and Weber, Nanninga and Mayor ([22]) substances have all hypotensive action. Moreover, kallikrein is a heat labile, protein like substance. V.D.M. has been identified with ferritin ([23]). It is a protein easy to differentiate chemically from angiodiactin.

Chemical structure. By its solubility in organic solvents, angiodiactin may be classified as a fatty substance. The fact that it precipitates from ethyl ether by sodium carbonate and from acetone by calcium hydroxide suggests the presence of an acidic group.

Action on arterial pressure. It is rather curious that angiodiactin being a vasodilator does not affect arterial pressure. Incidentally this also occurs with V.D.M., a vasodilator substance found in the liver. Although the mechanism involved is unknown, this dissociation between vascular and pressor effects could be explained in several ways such as: a) angiodiactin would be rapidly inactivated by the blood; b) cardiac output would increase and make up for vasodilatation; c) compensatory changes in other vascular areas; d) vasoconstrictor impurities, such as mentioned before, occurring in cruder preparations could modify pressor responses, etc. It is a well known fact that the place of action of a vasomotor substance on the vascular tree (arterioles, precapillaries, capillaries) greatly influences its action on arterial pressure. At present we do not know where the action of angiodiactin takes place.

Another possible explanation would be that angiodiactin is not a true vasodilator, but an inhibitor of vasoconstrictor substances.

SUMMARY AND CONCLUSIONS

Angiodiactin, a new vasodilator substance, has been described. Angiodiactin has been purified using the kidney as the starting material, but it has been found in the liver and probably exists in other tissues.

To demonstrate the vasodilator effect of angiodiactin, the toad vascular preparation, kept in a state of mild vasoconstriction by epinephrine, has been employed. Under these conditions tenths of a gamma of the most purified angiodiactin preparation so far obtained, produces an intense vasodilatation. The same effect can be obtained with angiodiactin adding hypertensin instead of epinephrine to the perfusing fluid, but in this case the vasodilator effect is slower and lasts longer.

Angiodiactin also shows vasodilator action on the rat vascular preparation constricted by epinephrine at doses of hundredths of a gamma.

Angiodiactin preparations are contaminated by a vasoconstrictor substance, which accompanies it during the various purifications steps. However most purified preparations have little or no vasoconstrictor effect.

Angiodiactin has no action on arterial pressure, toad's isolated heart or rabbit's isolated ileum.

Angiodiactin endures boiling, is soluble in fat solvents, and appears to have an acidic group.

Its chemical and biological characteristics allow to differentiate angiodiactin from other vasodilator substances.

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ON THE ABSORPTION MECHANISM OF PELLETS*

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THE absorption mechanism of pellets is not clearly understood. Some authors have suggested that insoluble materials disappear by phagocytosis (11, 17, 20). But the possibility still remains that highly hydrosoluble compounds would be dissolved in the tissue fluid before reaching the circulation (11, 19). Therefore, phagocytosis and solubility may play an important role in the absorption. This is emphasized by the fact that very soluble compounds are absorbed in few hours (13, 14, 15), while insoluble substances take several weeks (3, 7, 5, 11, 12). Moreover, it has been claimed that cholesterol delays absorption of the implanted substances and it has been demostrated also that cholesterol is reabsorbed in some instances while in others it is not (8, 16).

The object of this study was to learn about the role played by phagocytosis in the absorption mechanism of pellets and to study further the behaviour of cholesterol.

METHODS

The substances employed were the following: phenobarbital and phenobarbital with 70 % of cholesterol; papaverine hydrochloride; papaverine; theobromine and theobromine with 50 % of cholesterol; strychnine and strychnine with 50 % of cholesterol; testosterone and testosterone with 50 % of cholesterol; sulfadiazine; caffeine; cyclobarbital; morphine and cholesterol. Pellets —made by compression—, had a diameter of 3 mm and approximately the same weight (40 mg). When cholesterol was used, substances were dissolved in ether or finely blended in a mortar.

Some pellets were placed inside a cellophane bag tied by a thread. The capacity of the bag was slightly larger than the volume of the pellet. Both these sterilized pellets and cellophane bags —containing the pellets— were implanted aseptically in the subcutaneous tissue of unselected gui-

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nea pigs. Pellets and bags were removed some time after they were implanted. The removed pellets were dried out at 38°C. Loss of weight of the pellets was taken as an index of absorption. Cholesterol was assayed by using Liebermann's technique.

Some pellets were dialyzed at 38°C. The dialytic medium was: a) a solution of 7 % acacia gum in Tyrode without glucose, and b) horse blood serum. One ml of 0.1 % Merthyolate solution (Eli-Lilly) was added to 1 000 ml of the dialytic medium.

In some instances the implanted pellets and bags, together with the

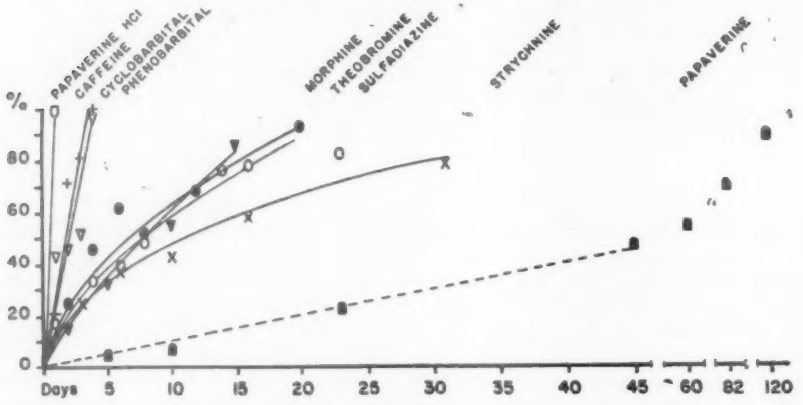

FIG. 1. — Absorption rate of pellets. *Each point represents the mean value of at least four different implantations.*

Papaverine HCl and caffeine have similar absorption rates. The absorption rate of papaverine was taken from Lewin, Melkonian and Vargas (12).

surrounding tissue, were fixed in formaldehyde, stained with hematoxylin-eosine and observed microscopically.

RESULTS

I. Absorption of unmixed drugs.

The implanted drugs were: papaverine hydrochloride, caffeine, cyclobarbital, phenobarbital, theobromine, morphine, strychnine and sulfadiazine. The solubility in water of these compounds is indicated in Table I.

The absorption rate of these pellets is illustrated in figure 1. In this, as in all the other cases, results represent the mean values of at least four different implantations.

II. Absorption of drugs mixed with cholesterol.

Phenobarbital with 70 % of cholesterol, theobromine and strychnine with 50 % of cholesterol were implanted. The absorption rate of these substances was slowed down by cholesterol, as shown in figure 2B. Cholesterol was not absorbed.

III. Absorption of unmixed drugs contained in a cellophane bag.

Every implanted bag contained one pellet each of the following drugs: caffeine, papaverine hydrochloride, phenobarbital, theobromine, papaverine and testosterone. The cellophane bag delayed the absorption of these compounds (figure 3).

The pellets of papaverine hydrochloride lost their cylindric shape and were transformed in a insoluble powder. Their absorption rate was very slow.

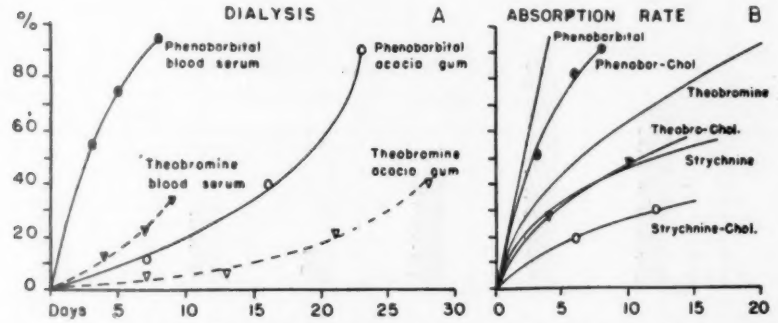

FIG. 2. — *A. — Comparison between the weight loss of pellets dialyzed against either blood serum or acacia gum solution.*
B. — Comparison between the absorption rate of implanted pellets with or without cholesterol.

IV. Absorption of drugs mixed with cholesterol contained in cellophane bags.

Pellets of phenobarbital-cholesterol (70 %) and testosterone-cholesterol (50 %) were employed. Cellophane bags did not modify the absorption of phenobarbital-cholesterol. The quantity of cholesterol was the same before and after the implantation. Furthermore, cellophane decreased the absorption rate of testosterone-cholesterol. In this case, cholesterol and testosterone disappeared from the pellet at the same rate.

V. Tissue reaction.

Caffeine, papaverine hydrochloride, phenobarbital (with or without cholesterol) and morphine produced slight inflamatory reactions. Strychnine induced great vascularization and hard swelling of the surrounding tissues. This phenomenon lasted about 20 days. Sulfadiazine produced oedema with important vascularization and macroscopic hemorrhage during the first 15 days. A noticeable oedema with great vascularization was the characteristic tissular reaction to theobromine. Something similar was observed after cyclobarbital, although of less intensity. All these reactions were attenuated by the presence of cholesterol.

The surrounding capsule was thicker when pellets were implanted inside cellophane bags. Cellophane itself did not induce any particular reaction.

No cellular elements could be demostrated inside the cellophane bags from 10 to 30 days after implantation.

VI. Dialysis of pellets.

A. — Pellets of papaverine hydrochloride, caffeine, phenobarbital, phenobarbital —70 % cholesterol, theobromine, testosterone, testostero-

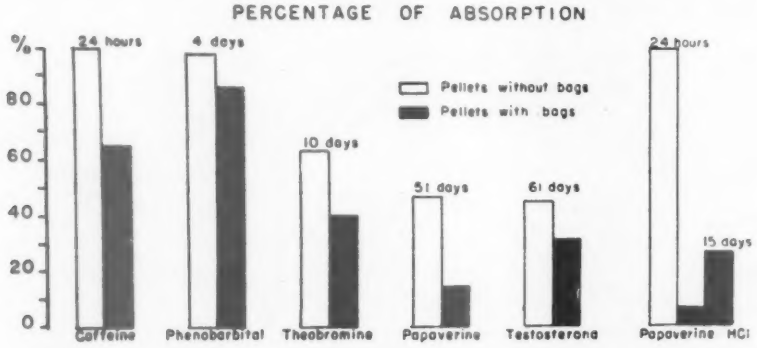

FIG. 3. — *Comparison between percentage of absorption of pellets contained in a cello phane bag and pellets not included in a bag.*

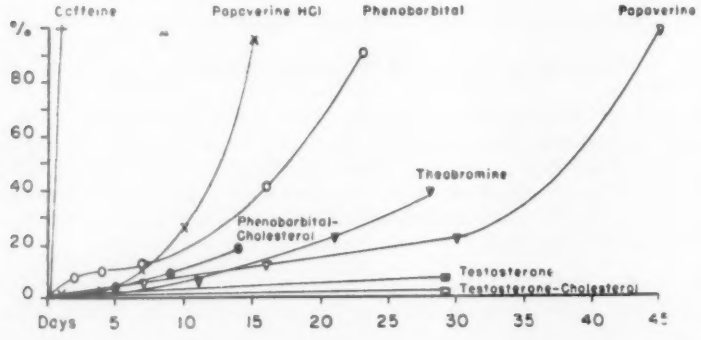

FIG. 4. — *Loss of weight of substances dialyzed against a 7 % acacia gum solution.*

ne— 50 % cholesterol, papaverine and cholesterol were dialyzed in 7 % acacia gum in Tyrode solution without glucose. All these pellets decreased in weight (figure 4) with the exception of cholesterol (up to 29 days of dialysis). It is important to emphasize the role played by cholesterol. When it was mixed with phenobarbital and with testosterone, cholesterol slowed down the loss of weight of these pellets.

B. — Pellets of phenobarbital and theobromine were dialyzed in hor-

se blood serum. Dialysis in this medium was faster than in Tyrode solution with acacia gum, as it is shown in figure 2A.

DISCUSSION

The difference in weight of pellets before implantation and after removal was taken as an index of absorption. This method is not very accurate because some strange material (protein, etc.) may be added during

TABLE I

Solubility of the employed substances

Substances	Water*	Tissue fluid**
Papaverine HCl	1:40	—
Caffeine	1:46	—
Cyclobarbital	1:500	—
Phenobarbital	1:1000	1:276
Theobromine	1:2000	1:750
Morphine	1:5000	1:835
Sulfadiazine	1:7700	1:850
Strychnine	1:6400	1:1100
Papaverine	—	1:2540
Testosterone	—	1:2590
Cholesterol	—	1:26300

* After The Merck Index. Sixth Edition. Merck and Co. Inc. Rahway. 1952.
** For details, see text.

implantation ([4, 6, 10, 17]). Nevertheless, the regular loss of weight and the lack of deformation of the pellets would indicate that the deposit material does not alter the absorption rate of pellets used in these experiments, such as happens when steroidal hormones are implanted ([6]).

A priori there are, at least, two possibilities to explain the mechanism of pellet absorption: a) dissolution in the tissue fluid and b) phagocytosis. When very hydrosoluble substances such as glucose, papaverine hydrochloride, caffeine, etc. are employed, the dissolution of the material in the tissue fluid and its passage into circulation would be the predominant factor. But with regard to practically insoluble compounds, dissolution would not play any role whatsoever. For this reason phagocytosis has been suggested as a mechanism of absorption. The presence of phagocytic cells in the tissue that surround the implanted pellet and the

presence of cholesterol and testosterone inside the phagocytic elements are the experimental bases for this hypothesis (17).

The index of hydrosolubility of various substances may differ with regard to their index of solubility in the tissue fluid and blood serum. The absorption rate of pellets practically insoluble in water such as papaverine and testosterone that were implanted in cellophane bags and the absence of phagocytic elements inside the bags suggest a dissolution of the substances in the fluid that has penetrated across the cellophane membrane.

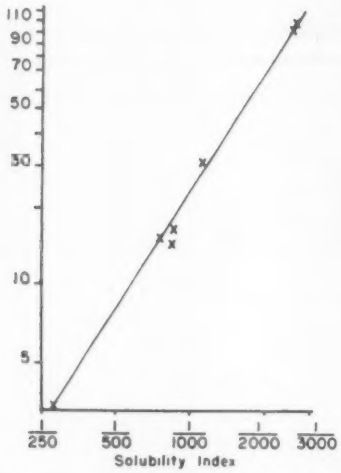

FIG. 5. — Relationship between solubility and absorption rate of the implanted pellets.
Abscissae: solubility in tissue fluid.
Ordinates: time in days necessary to absorb 80 % of the implanted pellets. The following substances were studied: Phenobarbital, theobromine, morphine, sulfadiazine, strychnine, papaverine and testosterone.

For this reason the solubility index of these compounds in a tissue-fluid-like medium was determined. The following technique was employed: phenobarbital, theobromine, morphine, sulfadiazine, papaverine, testosterone and cholesterol finely grounded were placed inside small cellophane bags which were weighted previously. They were dialyzed against a constant volumen of horse blood serum for 10 days at 38°C. under aseptical conditions. Differences in weight before and after dialysis would indicate approximately the solubility index (Table I).

A direct correlation between solubility in tissue fluid and absorption rate appears evident (figure 5). Two factors seem necessary to obtain this result: a) the compressed substance must not produce great injury to the tissue, and b) it must not induce considerable vascular changes. Oedema induced by theobromine during the first days of implantation

seems to increase the absorption rate; when oedema disappears, absorption becomes slower (figure 1). Protamine-zinc-insuline, isopropylnoradrenaline estearate, procaine-penicilline are not considered in this work although their absorption rate has been studied [9, 10, 18]. Their mechanism of absorption is not clearly understoood because it is not known whether splitting is necessary for absorption [1, 2].

The absence of phagocytic cells within the cellophane bag indicates that phagocytosis is not essencial for the absorption of the pellets. Moreover, the direct correlation between the solubility in a tissue-fluid-like medium and absorption rate suggests that absorption is performed by dissolution of the implanted material in the tissue fluid.

When substances are implanted inside cellophane bags the loss of weight is slower than when they are implanted without bags. The small amount of tissue fluid that trespasses the bag may slow down the absorption.

Cholesterol decreases the absorption rate of the implanted substances [16]. From our results, it may be roughly stated that the lower the solubility index, the greater is the absorption delay. Actually, very soluble compounds are not retarded [8, 13, 14]. The retarding action of cholesterol may be due to its hydrophobic properties. Furthermore, it has been demostrated that pellets of cholesterol are not absorbed after 90 days of implantation [8, 14]. Conversely, when cholesterol is mixed with either steroidal hormones or synthetic compounds with estrogenic activities, it is absorbed at a rate similar to that of the indicated substances [8, 15]. Since all these substances are liposoluble, it is possible that cholesterol may combine with them through physico-chemical forces. Thus, a new product would be formed with an intermediary solubility. Such possibility is reinforced by the fact that the weight loss of cholesterol pellets mixed with 50 % of testosterone (dialyzed in Tyrode with acacia gum) lies between pure cholesterol and testosterone. The delay induced by cholesterol in the dialysis of testosterone is approximately similar to the delay in the absorption rate observed with pellets of testosterone-cholesterol [16].

SUMMARY

Pellets of substances with different hydrosolubility index were implanted subcutaneously in guinea pigs; some were implanted after being placed inside cellophane bags. Other pellets were dialyzed against a solution of 7 % acacia gum in Tyrode without glucose, or against horse blood serum.

Results indicate that: a) Hydrosolubility and absorption rate could not be correlated.

b) Cholesterol delays the absorption rate of the compressed substances. Cholesterol was absorbed in some oportunities.

c) The absorption rate of pellets contained in cellophane bags was delayed but not abolished. No cells were found within the bags.

d) Dialysis rate was greater in horse blood serum than in a solution of acacia gum.

e) When solubility of the employed substances in a tissue-fluid-like medium was plotted against the absorption rate of the implanted pellets a clear correlation was found.

These phenomena are discussed and it is concluded that pellets implanted subcutaneously can be absorbed without the intervention of phagocytosis.

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MECHANICAL RESPONSE OF SKELETAL MUSCLE IN NORMAL, HYPERTHYROID AND HYPOTHYROID RATS*

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IN PREVIOUS experiments in the albino rat, Hoffmann et al. ([10]) observed that the thyroid hormone influences the responses of skeletal muscle to indirect stimulation. They showed that while thyroidectomy lengthens the time course of the muscular twitches, treatment with thyroxine has an opposite effect. This change in the time course of the twitch made it possible to obtain a fused tetanus with a low frequency of stimulation, in the thyroidectomized rats. In normal or hyperthyroid animals, the tetanizing frequency is markedly higher.

These early experiments were performed without controlling the body temperature of the animals, and the twitches were recorded with a semi-isometric spring lever.

It is a well known fact that the thyroid hormone plays some rôle in thermorregulation; one of the symptoms already emphasized in the early descriptions of myxoedema, is the tendency to hypothermia. Glaubach and Pick ([4]) have shown that the administration of thyroid hormone abolishes the drop in body temperature induced by the injection of phenobarbital in normal rabbits and guinea pigs. We observed that, at the same environmental temperature (around 18° C) hyperthyroid rats —anesthetized with pentobarbital— maintain their rectal temperature near 38° C., while in thyroidectomized rats, the temperature falls sometimes under 30° C. ([14]).

As it is widely known that low temperature lengthens the time course of muscular contraction, together with enhancing the development of tension ([11, 24]), a new series of experiments was performed under controlled rectal temperature, and recording semi-isometrically, both muscular twitches and tetanus using the photokymographic technique. These experiments confirmed the initial observations, although the differences between the normal, hyperthyroid and thyroidectomized groups of animals appeared diminished ([14]). These results seemed to agree with the

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observations of Lambert, Mederos and Kelsey [18] that muscular contractions evoked either by the ankle jerk or by "direct" electrical stimulation is much longer in myxedematous patients than in normal subjects. These responses were restored to normality by treatment with thyroid hormone.

Experiments by Díaz-Guerrero, Thompson & Hines [2] established that in rats treated with thyroxine, skeletal muscle develops less tension than in normal or hypothyroid rats. We had observed similar changes in our experiments, in which the twitch and tetanic tension developed by the triceps surae of hyperthyroid rats was significantly lower than the tension developed by normal or thyroidectomized animals. However, our results in these two last groups gave much higher absolute values for twitch tension than those obtained by Walker [22] for the same muscle in the albino rat. Our values for the tetanus-twitch ratio were around 2, while Walker reported an average ratio of 4.25. In view of these results it seemed likely that the high intensity and long duration of the electrical stimulus used by us, induced repetitive discharges, responsible for the higher tension, and possibly also for the longer rising and half relaxation times observed in our experiments.

The considerations expressed above, induced us to perform the experiments reported in this paper, in which the mechanical and electrical responses of the triceps surae of normal, hyperthyroid and hypothyroid rats to indirect stimulation, have been recorded under conditions of constant muscular temperature. The stimulation has been carried out with long series of indirect stimuli, at a frequency that would favour "contraction fatigue" [1].

This study, we thought, could perhaps contribute to the analysis of the influence of the thyroid hormone on neuromuscular transmission and the nature of muscular disorders observed under conditions of changes in thyroid function.

Methods

Male albino rats, weighing between 180 and 255 g were used. Hyperthyroidism was obtained by injecting thyroxine in daily doses of 10 μg/g of body weight, until the metabolic rate was 100 % above the normal level. This stage was thereafter maintained by reducing the daily doses of thyroxine to 5 μg/g. The experiments were performed after 27 ± 2 days of treatment, when the metabolic rate, which was measured indirectly every day (by the procedure previously described [12]) had reached a level of 88 ± 2.4 Cal/m²/h. In 9 animals, thyroidectomy was performed under ether anesthesia. The experiments in these animals were carried out 63 ± 7 days after the operation, when their metabolic rate had decreased to 31 ± 0.5 Cal/m²/h. In three animals, hypothyroidism was obtained by administration of thiouracil during 52 to 80 days, mixed with the food in a proportion of 0.50 g per kg, and dissolved in the drinking water in a concentration of 0.90 g per liter. The metabolic rate in these animals had descended to 33 Cal/m²/h when the experiments took place. Although oxygen consumption was not measured in the normal rats used as control, previous work has shown that their basal metabolic rate is of the order of 44 Cal/m²/h [14].

All the animals were anesthetized with sodium pentobarbital, injected subcutaneously. Normal and hypothyroid rats received 5-6 mg per 100 g of body weight; hyperthyroid rats, 3-4 mg/100 g.

Muscular temperature was measured with a copper-"constantan" thermocouple, placed deeply under the triceps surae, and connected to a

TABLE I

Triceps surae. Indirect stimulation with 12.5 stimuli per minute

Number	Animals	Contraction time msec.	½ relaxation time msec.	Peak tension g/g. muscle
		FIRST TWITCH		
7	Normal	14.0 ± 0.28	13.8 ± 0.80	505 ± 21.1
6	Hyperthyroid	14.2 ± 0.25	14.1 ± 0.30	465 ± 20.6
9	Thyroidectomized	14.3 ± 0.45	14.9 ± 0.76	510 ± 23.0
3	Thiouracil treated	14.1 ± 1.00	15.5 ± 1.76	565 ± 24.8
		SEVENTH TWITCH		Increase in tension with respect to first twitch
7	Normal	13.4 ± 0.33	11.7 ± 0.54	4 %
6	Hyperthyroid	13.6 ± 0.30	12.3 ± 0.45	3 %
9	Thyroidectomized	13.7 ± 0.47	12.9 ± 0.86	3 %
3	Thiouracil treated	13.4 ± 0.71	13.9 ± 1.40	0 %

Statistical analysis according to Günther (6).

mirror-galvanometer, which permitted an accurate reading of temperature differences of 0.1° Celsius. The other thermojunction was placed in boiling ether. The rectal temperature of the animals was maintained between 38°-39° C and the muscular temperature was adjusted at 38° C by radiant heating or ventilation of the surface.

The rats' left hind leg was firmly held by two steel pins: one passed through the femur at the insertion of the gastrocnemius and the other through the tibio-astragalin joint. Both pins were immobilized by a strong steel clamp. A bronze wire tied to the tendons connected the muscles to a strain-gauge as described by Libert (19). The electrical unbalance of the strain-gauge's Wheatstone bridge, induced by the bending of the steel plate during muscle contraction, was recorded with a Dumont dual-beam oscillograph, after passing through a differential condenser-coupled amplifier described by Grundfest (5)* (which possesses a sufficiently long

* We must express our acknowledgements to Dr. H. Grundfest for the construction of the stimulator and amplifiers, at the Department of Neurology, in Columbia University.

time-constant for an adequate recording of muscular twitches). The calibration of the strain-gauge was accomplished with weights, recording the electrical unbalance by a millivoltmeter. This instrument, which could be connected by a selector key to the strain-gauge, was used also to adjust the initial muscular tension, which was maintained throughout the experiments at 100 g, and also to test if during a period of repetitive stimulation, incomplete tetanus or contracture developed.

Muscle action potentials were recorded simultaneously with the mechanical responses on the other oscillographic beam, by leading from the

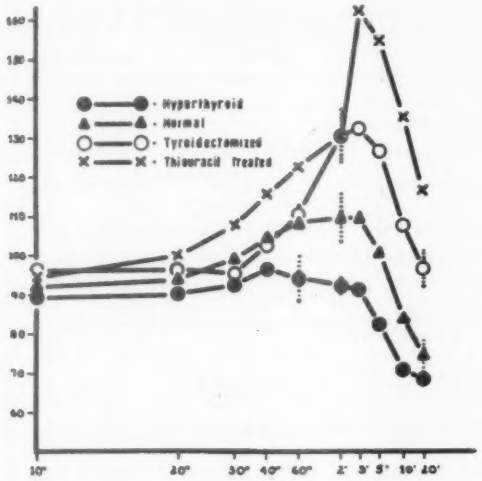

FIG. 1. — *Average duration of twitch contraction period. Triceps surae. Stimulation with 5.5 stimuli per second during 20 min. Abscissae: time of stimulation (logarithmic scale). Ordinates: percentage change of the duration; 100 % corresponds to the first twitch.*

steel pin that passed through the femur and a thin copper wire bound around the distal end of the muscle.

The muscle was stimulated indirectly with supramaximal intensity by rectangular electric pulses of 0.05 msec duration. The stimulator used was of the type described by Grundfest (ibid).

Both tension and duration of the muscular twitches were calibrated with a 100 cycle/second alternating current of 1 to 5 mV, connected to the amplifying channel used for the recording of the muscular contractions.

RESULTS

In each experiment two series of muscular contractions were recorded; one by indirect stimulation with a frequency of 12.5 per minute; the other with a frequency of 5.5 stimuli per second.

a) *Low frequency stimulation.*

In each experiment records of thirteen consecutive responses were taken, after a resting period of at least 5 minutes following the few isolated stimuli applied for the selection of supramaximal intensity. Table I

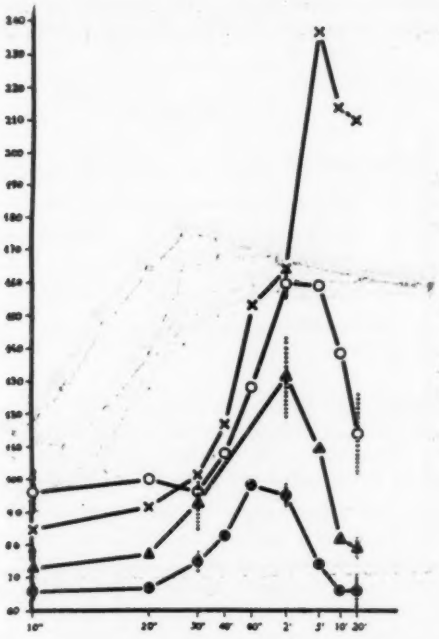

FIG. 2. — *Average duration of twitch half-relaxation period. Triceps surae. Stimulation, abscissae, ordinates and symbols, as in Fig. 1.*

shows the average rising time, half relaxation time and peak tension of the first and seventh twitches of the series. From the seventh to the thirteenth contraction no marked changes of the twitches were observed.

The results show no differences among the four groups of animals regarding the time course of the muscular twitches, but the strength of the twitch is significantly higher in thiouracil treated rats as compared with the hyperthyroid animals. It may be pointed out that in 6 thyroidectomized, one thiouracil treated and one normal rat, the muscle action potential was complex showing two definite peaks, separated by 2 to 3 milliseconds, as generated by repetitive responses or marked heterochronism of single responses (Fig. 4B). None of the hyperthyroid rats showed composite muscle action potentials.

b) *Stimulation with 5.5 stimuli per second.*

Stimulation with this frequency was maintained during a period of 20 minutes, the twitches being recorded at regular intervals.

Important differences in the time course and tension of the muscular contractions were observed under these experimental conditions, among the various groups of animals.

The contraction period of the first twitch has the same average duration in all groups, as shown in Fig. 1, and does not change during the

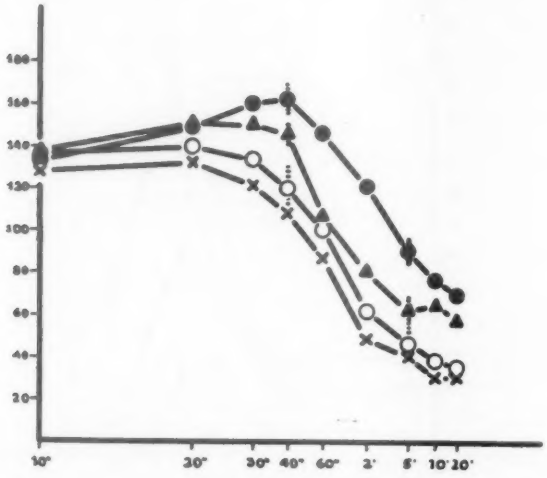

FIG. 3. — *Average twitch tension. Triceps surae.*
Stimulation, abscissae and symbols, as in Fig. 1.
Ordinates: percentage change of twitch tension; 100 % corresponds to the tension of first twitch.

initial 20-40 seconds of stimulation; but after this period, the contraction time increases in normal and especially in hypothyroid rats reaching a maximum in about 3 minutes, to decrease gradually thereafter. On the other hand, during the same period, the contractions of the hyperthyroid muscle do not show this lengthening but rather a progressive shortening of contraction time during the entire stimulation period. Differences of about 50 % in duration can be observed between the contraction time of hyperthyroid and thyroidectomized rats after 2 to 5 minutes of stimulation. The lengthening of the contraction time in the thiouracil treated rats is even greater than in the thyroidectomized animals. The chronological differences among the hyperthyroid, hypothyroid and normal rats are significant up to the end of the 20 minute period of stimulation. The

time course of the twitches of normal animals is intermediate to the thyroidectomized and thyroxine treated rats.

Regarding the duration of the half-relaxation time, significant differences were found between the first twitches of thyroidectomized and hyperthyroid rats. While in the first group the half-relaxation time does not show any important change, its duration is markedly shortened in normal and especially in hyperthyroid animals. As shown in Fig. 2, these differences persist during the initial 10 seconds of stimulation, but after 20-30 seconds relaxation tends to become slower in all groups, shortening

TABLE II

Maximum percentage increment of twitch tension during "treppe"

Normal	Hiperthyroid	Thyroidectomized	Thiouracil treated
55 ± 3.8	62 ± 6.1	44 ± 17.1	32 ± 6.8

again after 2-5 minutes. Throughout all these chronological changes of the half-relaxation time, the differences between normal, hyperthyroid and hypothyroid rats remain significant. The thiouracil treated animals, after 5 minutes of stimulation, showed an extremely slow relaxation.

As it may be observed in Fig. 3, a marked "staircase" or "treppe" phenomenon was present in all rats; tension increased from 30 to 60 % with respect to the first twitch. This increment persisted for about 1 minute in normal and hypothyroid rats, but persisted for 2-5 minutes in hyperthyroid rats. Although the maximum tension reached by the muscular twitches during "treppe" is high in hyperthyroid rats, the differences among the four groups are statistically significant only when the thiouracil treated rats are compared with the normal or hyperthyroid animals (table II).

After the period of "treppe" the tension developed by the individual twitches decreases gradually in all groups of animals, due to the onset of fatigue. The tension decrease is, however, more marked in thyroidectomized and thiouracil treated than in hyperthyroid animals; the normal rats follow an intermediate course between the former groups.

It is not possible to see, from our experiments, a clear relationship between the decrease in twitch tension during fatigue and the diminution of the corresponding muscular action potentials, because the changes of the latter are extremely variable in the different experiments. It seems likely that this variability depends on the inadequacy of the leads used. In the cases in which composite muscular potentials were observed, these dissappeared with the onset of fatigue.

After the 20 minutes period of stimulation it was possible, by con-

necting the strain-gauge to the millivoltmeter, to detect a residual tension, which was interpreted as being due to contracture; it reached an average value of 25 g in the thyroidectomized and of 125 g in the thiouracil treated rats. In normal and hyperthyroid rats contracture was observed only exceptionally and it never surpassed 20 g of tension.

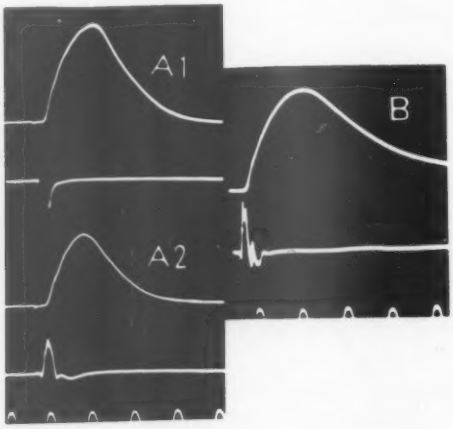

FIG. 4. — *Twitches and action potentials of the triceps surae.*
A_1 and A_2, hyperthyroid rat. Stimulation with 5.5 stimuli per sec.
A_1, first twitch.
A_2, twitch at the end of 20 min. stimulation.
B. — Hypothyroid rat; twitch recorded during stimulation with 12.5 stimuli per min.
Upper record, muscular contraction; lower record, muscular action potential.
Time: 10 milliseconds.

Fig. 4A, corresponds to muscular twitches of a hyperthyroid rat and shows that, as already described, after 20 minutes of stimulation with 5.5 stimuli/second the tension as well as the contraction and half-relaxation time are significantly decreased.

DISCUSSION

The preceding experiments clearly demonstrate that —if temperature is maintained constant— experimental hyper or hypothyroidism do not modify the time course of isolated twitches of the triceps surae of the albino rat, when stimulated indirectly at 12.5 stimuli/minute. However, continued stimulation during several minutes, with a frequency of 5.5 stimuli/second, results in lengthening of the contraction and half-relaxation times in hypothyroid animals, the differences among the groups being maximal at the end of 2 to 5 minutes of stimulation. After this

time, the duration of the contraction and half-relaxation periods decreases gradually in all groups.

Our results with low frequency stimulation do not agree completely with those reported by Lambert, Mederos and Kelsey [18] who observed that muscular contractions obtained by "direct" stimulation are much slower in myxoedematous patients than in normal subjects. These authors conclude that the differences are not due to the lower local temperature (0.5 to 1° C. below normal) of the hypothyroid muscle, since the temperature of normal muscles had to be lowered at least in 10° C. for the twitches to attain a similar duration as that observed in myxedema. As these authors performed their experiments on conscious subjects, with their muscles normally innervated, it could be argued that the existing tonus would tend to maintain the muscles in a condition more similar to a muscle stimulated with 5.5 stimuli/second than to a resting one with its motor nerve cut (the frequency of the nervous impulses responsible for normal muscular tonus being of the order of 5 to 10 per second [3]). Since Lambert et al. did not record muscular action potentials, the possibility that "direct" stimulation could have induced repetitive discharges in myxoedematous muscle can not be discarded.

Díaz Guerrero, Thompson and Hines [2] stimulated indirectly muscles of normal and thyroxine-treated rats with a frequency of 120/s, observing an increased muscular fatigability in the hyperthyroid group. On "direct" stimulation, however, this difference did not appear, a fact which indicates a possible influence of the thyroid hormone on the neuromuscular junction.

Our experiments using 5.5 stimuli/second show that, at least during 20 minutes, there is no evidence of increased fatigability in the hyperthyroid group compared to normal animals. In the hypothyroid rats, however, the decline in tension of the muscular twitches is much more marked than in the other groups. The great decrease in twitch tension in hypothyroid animals is partially more apparent than real, because of the tendency to tetanus due to the lengthening of the contraction and half-relaxation periods and the appearance of contracture after continued stimulation. These discrepances with the results reported by Díaz Guerrero et al. are probably due to the fact that —according to Rosenblueth [20] and del Pozo [1]— the low frequency of stimulation we used would produce "contraction fatigue" and not "junctional fatigue".

However, a certain percentage of "transmission fatigue" appears to be present, because of the gradual diminution of the height of the action potentials during prolongued stimulation with 5.5 stimuli/second. In this respect, it seems interesting to point that in the frog's sartorious, Steiman [21] has observed intermittent neuromuscular block, caused by fatigue, with stimulation frequencies as low as 5 per second.

The "treppe" seems to be a predominantly muscular phenomenon resulting from an increase in the contractile force of the muscle fibres, because Walker [23] observed no change in the muscular action potentials during its development at a stimulating frequency of 1/second. However, our results showing sometimes an increase in height of the action potentials during "treppe", suggest the possibility of a certain recruitment of inactive fibres, that would not be excited by a single stimulus, but require

repetitive stimulation. Recent experiments of Kuffler [16] in frog's muscle show that a single maximal stimulus applied to the nerve is incapable of bringing into action all the muscle fibres. The resting fibres can be excited by submitting the muscle to a certain degree of initial tension, or by repetitive stimulation, which would favour a summation of subminimal endplate potentials up to a threshold level. Hajdu and Szent-Györgyi [7] suggest that changes in the ionic permeability of the cardiac muscle membrane, modifying the intracellular ionic concentrations, may produce important changes in the magnitude of "treppe"; therefore it would seem advisable to perform parallel determinations of the intra and extracellular ion concentrations before attempting to explain the longer and higher "treppe" observed by us in the hyperthyroid rats, as compared to the thyroidectomized and thiouracil treated animals. Considering the influence of intravenous injections of KCl on the recovery of fatigued muscle [11] it might also be posssible that differences in intramuscular potassium or other ions may explain the different fatigability curves observed in hypo and hyperthyroid rats. The metabolic differences detected by Hoffmann and Horvath [15] in the skeletal muscles of hyper and hypothyroid rats may also contribute to explain the higher fatigability of the latter group, but it would be necessary to perform parallel mechanical and chemical determinations before venturing any conclusion.

The frequent ocurrence of muscular action potentials of a repetitive type observed in thyroidectomized rats, suggests that in hypothyroidism there exists a lowering of nerve accommodation, which would explain the tendency to repetitive nerve response.

The fact that, in the rat, thyroidectomy almost always leads to loss of a considerable portion of the parathyroid glands, might contribute to the appearance of composite action potentials, although, on the other hand, the observation of such potentials in thiouracil-treated rats and one normal animal seems to invalidate the possibility of hypocalcemia being an important factor in the production of the phenomenon in our experiments.

Another tentative explanation is suggested by a work of Hoffmann and Cori [13], who found that the psoas muscle of normal and thyroidectomized rats has a much lower threshold to adjacent intraarterial injection of acetylcholine than the same muscle of hyperthyroid animals. An increased reactivity of the end-plate region could be the cause of repetitive responses in presence of a normal quantity of acetylcholine liberated by the motor nerve fibres. In hyperthyroid rats, the lower excitability of the end-plates would prevent the induction of repetitive discharges.

There also exists the possibility that the type of composite action potentials could be the result of marked heterochronism in the responses of the various fibres of the triceps surae, which is a muscle of a rather complex structure.

Whether the composite action potentials observed by us, specially in hypothyroid rats, are due to repetitiveness or heterochronism, their nature may have some relationship with intramuscular potassium levels. Walker [22] has observed repetitive discharges in muscles of rats treated with desoxycorticosterone acetate and on a low potassium diet, conditions

in which the level of intramuscular K diminishes. The repetitive excitation disappears with exercise or by intraperitoneal injections of KCl. Composite action potentials were also observed by this author ([23]) in adrenalectomized rats, in which he found a higher twitch tension and longer contraction and half-relaxation times than in normal rats. However, he does not attribute to repetitiveness the alteration in the mechanical and electrical characteristics of the twitch.

Whatever may be the mechanisms of the repetitive muscular stimulation observed in our experiments, it seems most likely that this phenomenon does not intervene in the production of the increased tension and lengthened contraction time found in the muscles of hypothyroid animals, because of the brief time interval of the discharges.

As the disturbances in thyroid function produce important changes in the activity of the adrenal cortex, which plays such a fundamental rôle in the regulation of ionic metabolism, the muscular changes we have observed, could be actually induced through modifications of the intra and/or extracellular level of potassium ions, produced by the disturbance of the adrenal function.

Finally, we only mention the tendency of hypothyroid rats to develop contracture as a result of continued stimulation at a frequency of 5.5/second, without comenting on it, because the technique we used was not adequate for a careful quantitative study of this phenomenon.

SUMMARY

Muscular contractions of the triceps surae of the albino rat, stimulated indirectly with frequencies of 12.5 per minute, and 5.5 per second were comparatively studied in normal, hyperthyroid, thyroidectomized and thiouracil treated animals. Muscular twitches were recorded with a cathode-ray oscillograph, using the strain-gauge technique. Simultaneously the muscle action potentials were recorded. The experiments were performed at constant muscle temperature of 38° C. controlled thermoelectrically.

Apparently there exist no differences in the time course and the development of tension of the muscular twitches of normal, hypo and hyperthyroid rats when stimulated at a frequency of 12.5 stimuli per minute. However, during continued stimulation with higher frequency —5.5 stimuli per second— important chronological differences appear between hyperthyroid and hypothyroid rats. Contraction and half-relaxation times become shorter in the former, and longer in the latter, when compared with the first twitch obtained at the beginning of the stimulation period. The possible causes of these differences are briefly discussed.

In hypothyroid rats there exists a tendency to composite muscular action potentials, possibly due to repetitive or heterochronous responses of some muscle fibres. Stimulation with 5.5 stimuli per second produces, also, considerable "treppe", with an increment in tension which ranges from 30 to 60 % in the different groups of animals. "Treppe" is of much longer duration in hyperthyroid rats than in the other groups. The increment in tension observed during "treppe" is higher in hyperthyroid rats, but statistically significant only when compared with the thiouracil

treated animals. After "treppe" a gradual decrease in the peak tension of the muscular twitches is observed in all groups of rats. The fall in tension is significantly greater in hypothyroid than in thyroxine treated rats. When in the former group complex action potentials are observed, they become simple with the beginning of fatigue.

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THE INTERCHANGE OF CARBON DIOXIDE AND OXYGEN IN THE SKIN AND THE MUCOSAE OF HUMAN BEINGS*

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THE possibility of a gas transfer through the human skin has been studied since the last century. Gerlach (1856) and Schierbeck (1893) showed the existence of a small interchange; Krogh (1904), with better methods studied thoroughly the gas transfer through the skin of different phyla, his results in vertebrates being almost similar to those of Gerlach and Schierbeck (0.50 ml O_2 and 1.18 ml CO_2 per cm^2 per hour). However, the diffusion is very reduced in the skin of human beings. In Reptilia the transfer is negligible, but in other animals, such as Amphibia, due to the special disposition of the capillaries and to the nature of the skin, the gaseous interchange during resting conditions is performed mostly through this membrane (Krogh, 1904).

In consequence, we considered it of interest to test the gas transfer in normal human beings in structures without a corneal layer, such as mucosae and the skin in special conditions. Apart from its theoretical importance, this study could have a certain significance for the determination of dead space (Riley and Cournand 1949), and other pulmonary compartments.

METHODS

The skin of the periumbilical region was studied placing a Petri crystalizer of 10 cm diameter and 35 ml volume next to the skin. Its airtightness was checked with a water ring around its boundaries. The skin was washed in some experiments with alcohol and sulphuric ether and in others scraped with a razor blade, removing part of the corneal layer. The nasal epithelium was selected as representative of cylindric

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ciliated structures and the prepuce and glans as squamous epithelium. In order to obtain a closed system in the nose one choane was obstructed with a water filled bladder and the corresponding nares with a rubber stopper which was provided with two catheters, one connected with the bladder and the second for obtaining gas samples (Fig. 1). After obstructing the posterior choane, the nasal cavity was thoroughly ventilated with atmospheric air. It was not difficult to maintain the aistightness, but the bladder produced a certain amount of pain and discomfort which precluded the continuance of the experiments for more than 40 minutes at a time.

Fig. 1

Regarding the prepuce and glans, after cleaning with soap and occasionally alcohol, from 6 to 8 ml were insuflated through a glass tube provided with rubber coating tied to the skin of the prepuce.

The gas analysis were made in duplicate in a Scholander microgasanalyzer. The subjects used were normal. Two men and one woman were submitted to numerous experiments.

RESULTS

Skin. — The gas analysis of unwashed periumbilical skin gave similar results after 30 minutes in men and woman. The CO_2 percentage averaged 0.28 ± 0.05 and the O_2 20.72 ± 0.07. When the skin was washed with ether and alcohol the CO_2 rose to 0.80 and the O_2 was down to 20.23 approximately at the end of 30 minutes. In the skin washed, shaved, and with a thin corneal layer removed —without producing hemorrhage— the CO_2 percentage was 0.85 and 0.88 respectively in two experiments and the O_2 20.36 and 20.30, which corresponds to an interchange around 0.005 ml of O_2 per cm^2 and per hour.

In order to compare another region with particular skin characteristics the skin of the scrotum was used. In three 30 minute experiments in the same subject the values for CO_2 were 0.89, 0.94 and 0.96 and for O_2 20.23, 20.11 and 20.11. But although the gas volume is similar to the one of the Petri capsule the different surface areas do not allow comparison.

Mucosae. — The nasal mucose was studied with the above mentioned technique. The variations in relation to the time of the nasal obstruction obtained in the most studied subject are seen in Table I. In the other subject the results were similar in two experiments. Examinations of the right and left nasal cavity gave different results in the same subject, but they were done on successive days.

TABLE I

Time in min.	CO_2 %	O_2 %	$\frac{CO_2}{O_2}$
3	3.37	18.30	1.28
4	3.46	17.73	1.04
10	5.24	16.31	1.13
15	5.72	16.52	1.29
22	5.84	15.32	1.04
30	5.92	15.20	1.02
40	6.15	13.38	0.81
40	6.24	13.40	0.82

When ventilation of the nasal cavity with atmospheric air was performed following the respiratory rhythm of the subject and with a minute ventilation of 27 and 63 ml respectively, the results of the air samples obtained in two experiments were: CO_2 0.54 % and O_2 20.35 %; CO_2 0.37 % and O_2 20.57 %. The CO_2 elimination per minute was thus 0.14 and 0.20 ml and the absorption of O_2 was 0.16 and 0.23 ml respectively.

Mucose of the glans and prepuce. — Samples contained in the cavity formed by the prepuce were taken in experiments realized on successive days in the same subject at different intervals (Table II).

Discussion

A certain amount of gas interchange between the air and the tissues by simple diffusion should be accepted according to the figures given by Krogh (1904). However, the diffusion process through the connective

tissues being a million times slower than in air, it might be supposed that the quantities of gas diffused are minimal or that the degree of utilization of the interposed cells is higher than the diffusion rate. The results obtained from the skin show a more reduced gas transfer than those registered by Gerlach (1856), Schierbeck (1893) and Krogh (1904) and show that the skin forms an almost complete barrier. The passage is only slightly facilitated by the removal of grease and of a fine film of epidermis. On the contrary, in the nasal cavity the intensity of the interchange

TABLE II

Time in min.	CO_2 %	O_2 %	$\frac{CO^2}{O^2}$
10	1.96	19.63	1.50
15	3.65	19.12	1.29
20	3.68	18.33	1.41
40	5.16	16.47	1.15
50	5.31	16.24	1.13
60	5.80	14.80	0.92

is surprising, due probably to the great vascularization of the nasal mucose, the thinness of the epithelium and its relatively large surface. Not counting the neighbouring cavities, the volume of the nasal fossa of the most studied subject, measured between the posterior bladder and the stopper of the nares, reached 9 ml. Added to the 4 ml contained in the syringe the total amount of CO_2 liberated and O_2 absorbed can be calculated. Actually these are so small that they are negligible in assisting the pulmonary function, but if related to the surface of the nasal cavity they can be compared with the alveolo-capillary epithelium. The surface of the latter is around 60 m^2 (Willson, 1922; Müller, 1945, etc.) which, with an O_2 consumption of 200 ml per minute represents 0.0003 ml of O_2 absorbed per cm^2 per minute. The calculation of the surface of the nasal cavity was only approximate —around 30 to 40 cm^2— which represents an O_2 consumption of about 0.005 ml per cm^2 per minute. Naturally, the comparison has only a relative value because of the different gradients of the gas tensions in both systems.

The experiments of Henderson and Stehle (1919) in human beings showed that a certain gas transfer occurred in the oral cavity. However their experiences were not under "air tight" conditions and they studied the time that a tensional equilibrium was reached between air mixtures of different concentration. With regard to the CO_2 they established a percentage of 7.5, but the O_2 diminished always even starting with as low a mixture as 0.50 %. In this respect it is timely to remember that the richness of bacterial flora of the mouth can act similarly to the cells and bacteria in closed piopneumothorax (Matzuzawa, 1939; Soubrié and La-

bourt, 1942), where the O_2 level reaches less than 0.10 %. In the above mentioned studies of Henderson and Stehle the progressive fall in O_2 could be interpreted as a result of greater utilization than diffusion rate.

The other point worth mentioning is the greater diffusion velocity of CO_2 than that of O_2 in short experiments. As CO_2 is more diffusible in water solutions than O_2 it is easy to understand the initial CO_2/O_2 ratio. Later, the CO_2 excretion is slower, once the CO_2 tension begins to equal that of the CO_2 tension of the capillary venous blood, which is accomplished in the nasal cavity after approximately 30 minutes. On the contrary, the O_2 absorption after 30 minutes is greater than the CO_2 absorption since at that time the pressure gradient of O_2 of the nasal cavity is maintained and compensates the greater diffusion velocity of CO_2 in animal tissues.

It is remarkable that in the two experiments with ventilation of the nasal cavity the ratio CO_2/O_2 was less than 1 when in the closed system a quotient of 1.28 was registered after three minutes. Galdston and Horwitz (1948), found, in experiments performed in the supraglottic portion, that the interchange of gas in the dead space cannot explain the higher respiratory quotient of the expired air in regard to the alveolar air, as assumed by Henderson, Chillingworth and Whitney (19.5). They pointed out that during apnea the concentration of CO_2 increases progressively, while the O_2 diminishes only in the first ten seconds. This fact, unexplained by the authors, coincides with the results of our experiments of nasal ventilation.

Finally, the absolute quantities of gas that could diffuse through the respiratory conducting airways reveal that the concept of respiratory epithelium given to the alveolo-capillary membrane is only quantitative. It is natural to assume that the thinner the cylindric epithelium of bronchi becomes, cuboidal in bronchioli, the more the gas diffusion increases. sion of gases and because the mixture of alveolar air and dead space air is then not feasible, not only on account of the intrachanalicular diffusion of gases and because the mixture of alveolar air and dead space air in expiration eliminates a square front (Fowler, 1948), but also on account of the gas transfer through the walls of the smaller airways.

SUMMARY

In normal human beings the gaseous interchange through the skin is minimal, its amount being 0.005 ml of O_2 absorbed per cm^2 per hour. In nasal epithelium the gas transfer is much more intense, reaching 0.005 ml per cm^2 and per minute; and in prepucium and glans a little less than in nasal epithelium. The causes that produce a ratio CO_2/O_2 higher than 1 in the first minutes of the experiments and its posterior reversion are discussed. The gaseous interchange through the walls of

the smaller airways, the intrachanalicular diffusion, and the mixtures of alveolar air and the dead space volume in expiration eliminates a sharp limit between alveolar air and dead space. The concept of respiratory epithelium becomes a quantitative one.

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milímetro	mm	mililitro	ml	por ciento	%
micrón	μ	kilogramo	kg	hora	h
milimicrón	mμ	gramo	g	minuto	m
Ansgtröm	A	miligramo	mg	segundo	s
				milisegundo	ms

Para evitar la confusión derivada de la notación decimal diferente según los países, se adopta el punto decimal y se suprime toda notación entre millares sustituyéndose por un espacio: 10 000 (no 10.000 ni 10,000) — 0.90 (no 0,90).

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